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**14. ABSTRACT**

BRAF mutations are present in over half of all melanoma tumors. Although BRAF inhibitors (BRAFi) elicit rapid anti-tumor responses in the majority of patients with mutant BRAF$^{V600E}$ melanoma, the tumors inevitably relapse after a short time and are resistant to further treatment with these drugs. Thus, new therapeutic strategies are urgently needed to overcome the acquired resistance to BRAF inhibitors. Our goal is to test whether melanomas, which develop resistance to BRAF inhibitors, can be successfully treated using a novel arylpolyamine (***AP***) drug that is cytotoxic upon cellular internalization via the polyamine transport system (PTS). We found that human and murine BRAF$^{V600E}$ melanoma cells demonstrate greater PTS activity and increased sensitivity to ***AP*** compared to BRAF$^{WT}$ melanoma cells. Treatment with DFMO upregulates PTS activity in BRAF$^{V600E}$ melanoma cells and further increases their sensitivity to ***AP***. Human BRAF$^{V600E}$ melanoma WM983B-BR subline with acquired BRAFi resistance (following chronic *in vitro* exposure to the BRAFi, PLX4720) demonstrated increased PTS activity compared to the parental BRAFi-sensitive WM983B cells. Preliminary animal tumor studies have revealed that co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** plus DFMO delays the recurrence of BRAF$^{V600E}$ melanoma tumors that occurs in animals treated with PLX4720 alone.

**15. SUBJECT TERMS**
Melanoma, polyamine transport system, BRAF mutations, CXCR4, cancer stem cell populations, BRAF inhibitor resistance, macrophages

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## 1. INTRODUCTION:

Melanoma is a highly aggressive tumor with poor prognosis in the metastatic stage. Multiple oncogenic mutations (including BRAF, NRAS, KIT) drive this highly heterogeneous disease, with BRAF mutations detected in half of all melanoma tumors. Although targeting the V600E-mutant BRAF kinase with BRAF inhibitors significantly improves survival of patients with metastatic melanoma, recurrences often occur within several months. Development of BRAFi-resistance enriches for highly tumorigenic and metastatic melanoma initiating cells (MIC) or cancer stem cells (CSC) and increases tumor-promoting macrophages. CSC survival requires increased cellular uptake of polyamines via activation of the polyamine transport system (PTS). Our **objective** is to exploit the oncogene-induced PTS activity in metastatic melanoma cells by targeting the PTS with a novel arylmethyl-polyamine (***AP***) compound which is cytotoxic upon cell entry. Both exogenous polyamines and polyamine-based drugs are imported into tumors via this specific polyamine uptake system. However, normal cells are predicted to be significantly less sensitive to ***AP*** since they have low PTS activity. Recently we observed that another polyamine-containing compound, AMD3100 (an inhibitor of chemokine receptor CXCR4 and tumor invasiveness), weakly inhibits PTS activity in tumor cells. CXCR4 signaling in CSCs is necessary for tumor metastasis. The rationale for our studies is that ***AP*** will not only inhibit uptake of polyamines but will also inhibit CXCR4/SDF-1 signaling that is a critical regulator of melanoma-stromal interactions driving metastasis. **We hypothesize that *AP* 1) will kill BRAFi-resistant melanoma CSCs as a result of induced PTS activity and 2) will block CXCR4 signaling in metastatic tumor cells and stromal macrophages, thus inhibiting melanoma progression and metastasis.** The proposed research is novel in that it explores the association between polyamine transport and CXCR4/SDF-1 signaling in the survival of CSC subpopulations, metastasis, and BRAFi resistance. Another important novel aspect is that ***AP*** may also target M2 macrophages that highly express CXCR4 and have been found to contribute to BRAFi resistance and to indirectly facilitate melanoma progression and metastasis. To test our hypothesis, we have proposed the following specific aims:

**1)** to compare human BRAFi-sensitive and BRAFi-resistant melanoma tumor cells for effects of ***AP*** on spheroid-forming capacity; cell death and autophagy markers; invasiveness thru collagen-coated filters towards SDF-1; PTS activity (measuring the $V_{max}$ of $^3$H-spermidine); and melanoma tumor cell survival in macrophage co-culture assays ± PLX4720; and

**2)** to evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth and metastasis in mice following orthotopic injection of mutant BRAF melanoma cells.

## 2. KEYWORDS:

Melanoma, polyamine transport system, BRAF mutations, CXCR4, cancer stem cell populations, BRAF inhibitor resistance, macrophages

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

**Aim 1:** To compare the effect of increasing concentrations of ***AP*** with long-term and pulse BRAFi (PLX4720) co-treatment a) on the enrichment of BRAFi-resistant slow cycling CSC melanoma subpopulations and b) in melanoma tumor cell survival in macrophage co-culture assays ± PLX4720.

**Major Task 1:** To investigate the extent to which PTS activity and ***AP*** cytotoxicity correlate with sensitivity to BRAFi in human melanoma cell lines that have been treated chronically with PLX4720 (Months 1-14)

**Milestone Achieved:** Determination of the effect of BRAFi resistance on PTS activity and ***AP*** cytotoxicity in melanoma cells (Month 14)

We have obtained a series of human melanoma cell lines from Dr. Meenhard Herlyn (The Wistar Institute) that are $BRAF^{WT}$ or $BRAF^{V600E}$. We expanded the cells, made frozen stocks, and verified their sensitivity (or resistance) to the BRAF inhibitor (BRAFi), PLX4720 via proliferation assays and determinations of $IC_{50}$s. BRAFi-sensitive and BRAFi-resistant cells were assayed in the presence of varying doses of ***AP*** with and without 1 mM DFMO to determine the $IC_{50}$ for ***AP*** and the uptake of $^3$H-spermidine as a measure of their polyamine transport system (PTS) activity. We have accomplished about 80% of this major task, with our results reported in a poster presentation at the Polyamines Gordon Research Conference in June, 2017.

**Major Task 2:** To evaluate the effect of increasing concentrations of ***AP*** on CSC subpopulations that are enriched following chronic BRAF inhibition (Months 5-16)

**Milestone Achieved:** Determine to what extent ***AP*** can eliminate CSC-like subpopulations that are enriched following chronic BRAF inhibition (Month 16)

To address this task, we have taken advantage of WM983B melanoma cells that are BRAF mutant and PLX4720 sensitive and a WM983B-R subline that was generated by culturing in increasing concentrations of PLX4720 for several weeks to achieve a PLX4720 resistant subline. Although we have found that PTS activity is significantly elevated in WM983B-R cells compared to parental WM983B cells, we have not found any evidence that CSC subpopulations are enriched in WM983B-R cells following chronic BRAF inhibition with PLX4720 compared to that in parental WM983B cells. For that reason, we have shifted our focus to experiments outlined for major task 3 with about 25% completion of major task 2. Experiments in task 3 use a different approach using a shorter high dose treatment with the BRAF inhibitor PLX4720 to enrich for a CSC subpopulation that can be identified by a GFP reporter gene driven by the promoter of the CSC marker gene, JARID1B.

**Major Task 3:** To evaluate the effect of increasing concentrations of ***AP*** on the slow cycling JARID1B$^+$/EGFP$^{high}$ subpopulation that is enriched following a 24-96 hr pulse PLX4720 treatment of mutant BRAF melanoma cells transduced with a JARID1B-promoter-EGFP-reporter construct (Months 8-18)

**Milestone Achieved:** Determine to what extent ***AP*** can overcome the enrichment of slow cycling, invasive J/EGFP$^{high}$ BRAF inhibitor-resistant melanoma cells and its correlation with PTS activity and CXCR4 signaling. (Month 18)

We have begun to characterize two human BRAF$^{V600E}$ melanoma cell lines that have been transfected with a GFP reporter gene that is driven by the promoter of the JARID1B gene, a CSC marker. Wm3734$^{JARID1Bprom\text{-}EGFP}$ cells and 1205Lu$^{JARID1Bprom\text{-}EGFP}$ cells have been treated with a BRAFi (using a high concentration of 25 μM PLX4720) without or with increasing concentrations of ***AP*** for up to 72 hr. These cells have been cultured and analyzed using a 3-dimensional spheroid model and also as a standard 2-dimensional monolayer culture. We have completed 50% of our proposed experiments for task 3.

**Major Task 4:** To evaluate to what extent ***AP*** will block macrophage-mediated resistance to treatment with BRAF-inhibitors (Months 8-18)

**Milestone Achieved:** Determine to what extent ***AP*** can block the ability of macrophages to confer resistance to BRAF inhibition in melanoma cells. (Month 18)

Monocytes purified from human donor peripheral blood have been differentiated to macrophages using human melanoma conditioned medium or by adding cytokines (GM-CSF or M-CSF). We have confirmed the observation that M2-macrophages contribute to the melanoma tumor cell resistance to the BRAFi, PLX4720, using a human macrophage and melanoma cell co-culture system. We have completed 50% of task 4 experiments.

**Aim 2.** To evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth and metastasis in mice following orthotopic injection of mutant BRAF melanoma cells.

**Major Task 1*:*** To determine the dosing scheme of ***AP*** to be used in murine tumor studies (Months 1-8)

**Milestone Achieved:** Determine an ***AP*** dosing protocol for testing in mouse models of melanoma (Month 8)

IACUC and ACURO approval for our proposed mouse experiments with ***AP*** was obtained in the first couple months of this grant. We have completed task 1 to determine an ***AP*** dosing protocol in mice bearing either B16F10 tumors or YUMM1.7 melanoma tumors.

**Major Task 2:** To determine to what extent ***AP*** will increase the anti-tumor activity of PLX4720 following s.c. injection of BRAF mutant melanoma tumors in mice (Months 6-24)

**Milestone Achieved:** Determination of anti-tumor effect of ***AP*** treatment using melanoma xenografts *in vivo*, its correlation with sensitivity to BRAF inhibitors, and the extent to which combination ***AP*** and PXL4720 may overcome BRAFi-resistance and promote tumor killing. (Month 24)

IACUC and ACURO approval for our proposed mouse tumor experiments with ***AP*** was obtained in the first couple months of this grant. Our initial animal tumor studies in mice subcutaneously injected with YUMM1.7 melanoma cells have revealed that co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** plus DFMO delays the recurrence of $BRAF^{V600E}$ melanoma tumors that occurs in animals treated with PLX4720 alone. We have completed 33% of task 2.

**Major Task 3:** Statistical analysis of data; prepare figures and manuscript(s) to report results of the study (**Gilmour and Phanstiel, Sites 1 and 2**, Months 13-24)

**Milestone Achieved:** Manuscript(s) submitted for publication. (Month 24)

Collection of data, preparation of figures, and statistical analyses has been ongoing. No publications have been submitted for publication. However, some data were presented in a poster presentation at the Polyamines Gordon Research Conference in June, 2017.

**What was accomplished under these goals?**

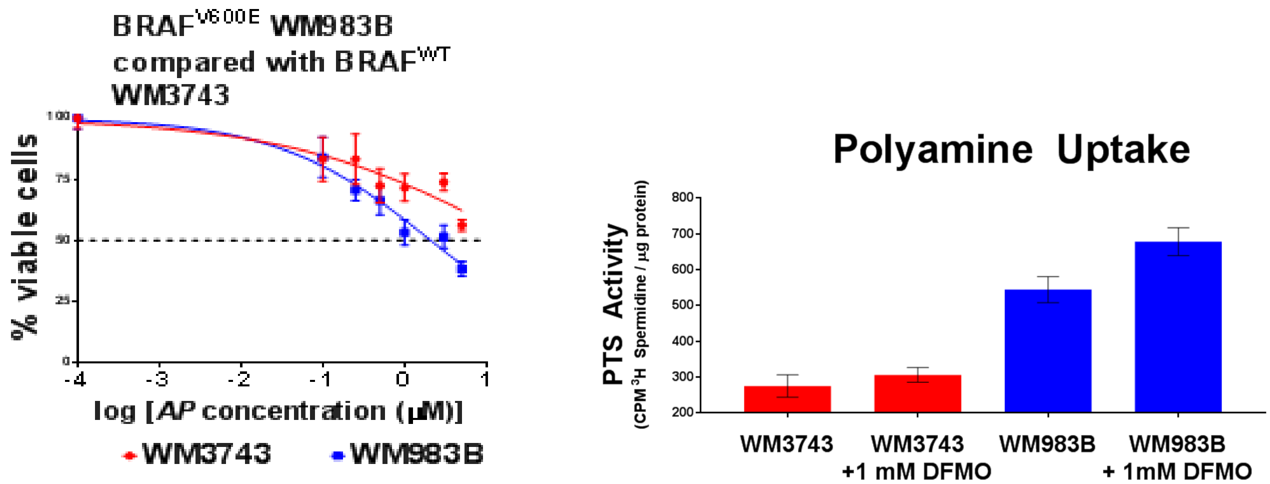
***Summary of Significant Findings:*** PTS activity (assayed via spermidine uptake following a 60 min pulse with 1 μM [$^3$H]spermidine) was significantly elevated in BRAFi-resistant melanoma cells compared to the parental BRAFi-sensitive cells. In addition, $BRAF^{V600E}$ melanoma cells are significantly more sensitive to ***AP*** (lower IC50) compared to $BRAF^{WT}$melanoma cells. Treatment with DFMO upregulates PTS activity in $BRAF^{V600E}$ melanoma cells and further increases their sensitivity to ***AP*** (lower IC50). Preliminary animal tumor studies show that co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** plus DFMO delays the recurrence of $BRAF^{V600E}$ melanoma tumors that occurs in animals treated with PLX4720 alone.

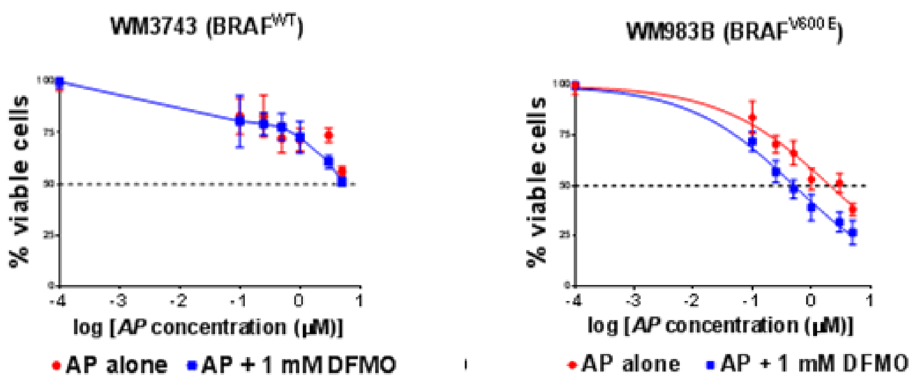
**Aim #1, Major Task 1:** Effect of BRAFi resistance on PTS activity and ***AP*** cytotoxicity in melanoma cells

We have spent considerable effort in obtaining and characterizing 18 different melanoma cell lines (15 human melanoma cell lines and 3 murine melanoma cell lines) for their BRAF mutational status; sensitivity (IC50) to the BRAFi, PLX4720; sensitivity to ***AP*** ± DFMO; and their polyamine transport system (PTS) activity as determined via spermidine uptake following a 60 min pulse with 1 μM [$^3$H]spermidine in the absence and presence of DFMO. Human melanoma cells were obtained from Dr. Meenhard Herlyn (The

Wistar Institute) and BRAF$^{V600E}$ YUMM1.7 murine melanoma cells from Dr. M. Bosenberg (Yale Medical School). We wanted to determine whether 1) PTS activity is increased in BRAF$^{V600E}$ melanoma cells compared to BRAF$^{WT}$ melanoma cells; 2) to what extent blocking polyamine biosynthesis with difluoromethylornithine (DFMO) would increase PTS activity; and 3) to what extent sensitivity to ***AP*** correlated with changes in PTS activity. In addition, we tested our hypothesis that BRAFi-resistance of melanoma tumors correspond to a high demand for polyamine growth factors and upregulated PTS activity. This was complicated by our observation that the PTS activity is greatly affected by the confluency of the cells and also the highly variable plating efficiency and growth rates of the different cell lines. We standardized our assays so that the cells were 80% confluent within a similar protein range to which the [$^3$H]spermidine uptake was normalized. Examples of some of our significant findings are summarized in the following figures.

**Fig. 1. Greater PTS Activity and Increased Sensitivity to *AP* in BRAF$^{V600E}$ Human Melanoma Cells Compared to BRAF$^{WT}$ Cells**

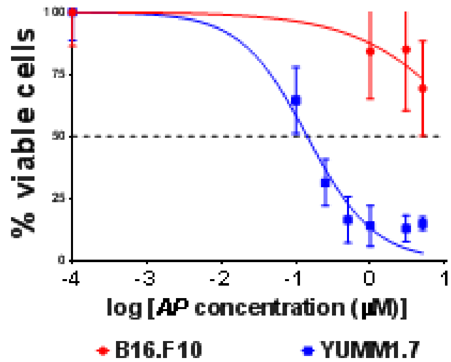


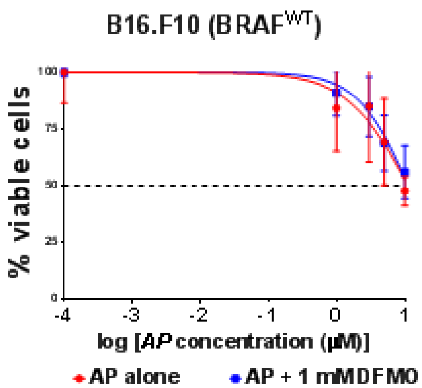

| Cell Line | *AP* IC50 (μM) | *AP* + 1 mM DFMO IC50 (μM) |
|---|---|---|
| WM3743 | 25.23 | 7.431 |
| WM983B | 2.004 | 0.5127 |

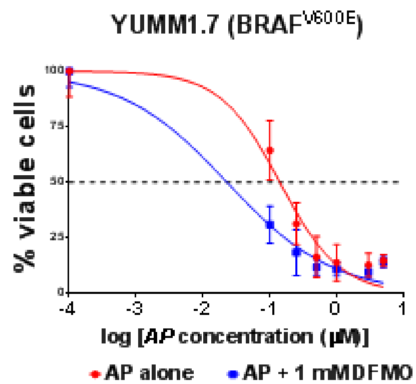
We found that BRAF$^{V600E}$ melanoma cells are significantly more sensitive to ***AP*** (lower IC50) compared to BRAF$^{WT}$melanoma cells. This is illustrated in Fig. 1 comparing WM3743 cells (BRAF$^{WT}$) with WM983B cells (BRAF$^{V600E}$) that shows a much lower IC50 for ***AP*** in WM983B cells which reflects the higher PTS activity compared to that in WM3743 cells. Cells with higher PTS activity will not only transport in more polyamines but also the polyamine-containing drug ***AP***. Treatment with DFMO not only blocks polyamine biosynthesis but also further upregulates polyamine uptake in many tumors via the PTS. As a result, DFMO treatment further increased the sensitivity to ***AP*** (lower IC50).

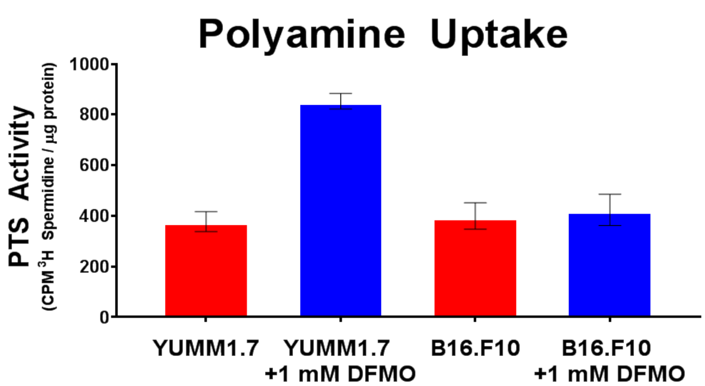
**Fig. 2. Murine BRAF$^{V600E}$ YUMM1.7 Melanoma Cells are More Sensitive to *AP* than BRAF$^{WT}$ B16F10 Melanoma Cells**




| Cell Line | PLX4720 IC50 (µM) | AP IC50 (µM) | AP + 1 mM DFMO IC50 (µM) |
|---|---|---|---|
| B16.F10 | 5.88 | 13.41 | 11.46 |
| YUMM1.7 | 1.59 | 0.1417 | 0.02299 |

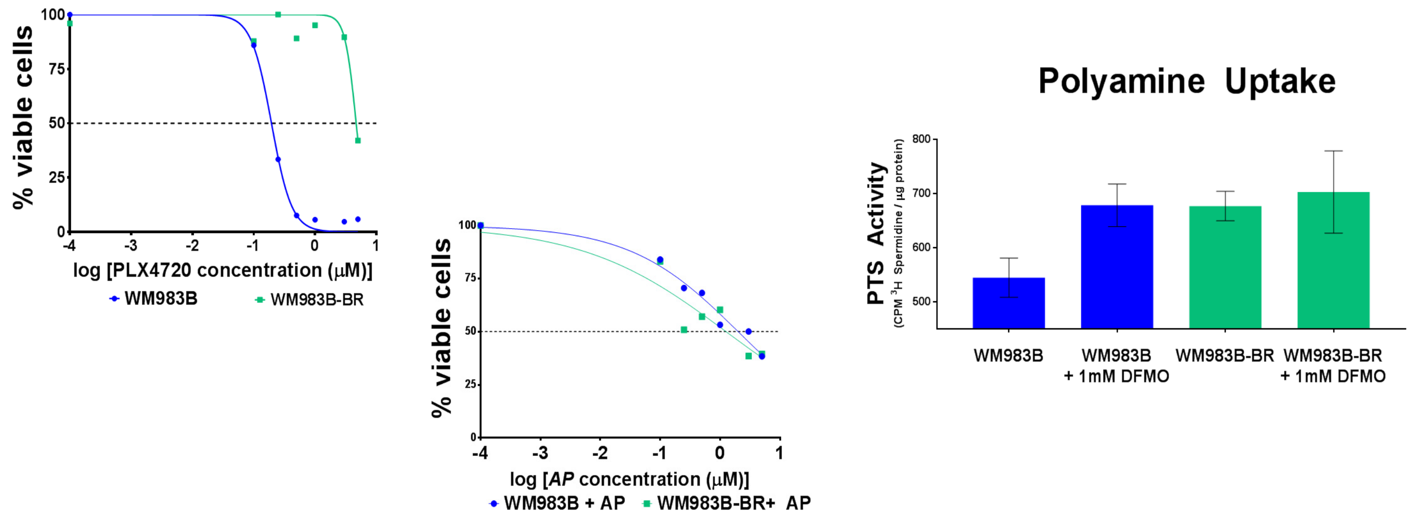



We found similar results when comparing murine melanoma cell lines B16F10 (BRAF$^{WT}$) and YUMM1.7 (BRAF$^{V600E}$). As expected, YUMM1.7 cells are very sensitive to the BRAFi, PLX4720 with a low IC50 compared to B16F10 cells. However, we also found that BRAF$^{V600E}$ YUMM1.7 cells are significantly more sensitive to ***AP*** with a much lower IC50 for ***AP*** compared to that found with BRAF$^{WT}$ B16F10 cells. In particular, DFMO co-treatment dramatically increases the sensitivity of YUMM1.7 cells to ***AP***, and this correlates with a huge DFMO-induction of PTS activity in BRAF$^{V600E}$ YUMM1.7 cells. These results correlate with our initial *in vivo* tumor experiments where we have found that co-treatment with both DFMO and ***AP*** result in less adverse effects in YUMM1.7 tumor-bearing mice, greater accumulation of ***AP*** in the tumor, and greater anti-tumor efficacy.

**Fig. 3. Human BRAF$^{V600E}$ Melanoma WM983B-BR Subline with Acquired BRAFi Resistance Demonstrates Increased PTS Activity Compared to Parental BRAFi-Sensitive WM983B Cells**




| Cell Line | PLX4720 IC50 (μM) | *AP* IC50 (μM) | *AP* + 1 mM DFMO IC50 (μM) |
|---|---|---|---|
| **WM983B** | 0.1944 | 2.004 | 0.5127 |
| **WM983B-BR** | 4.679 | 1.258 | 2.42 |

We compared WM983B cells with its subline WM983B-BR which has acquired BRAF inhibitor resistance via chronic *in vitro* exposure of WM983B cells to 1 μM PLX4720. The basis for our study is that the oncogene addiction and BRAFi-resistance of melanoma tumors correspond to a high demand for polyamine growth factors and a greatly upregulated PTS activity. We were encouraged to find that basal PTS activity is indeed greatly increased in the WM983B-BR cells that can grow in 1 μM PLX4720 compared to the parental, PLX4720-sensitive WM983B cells. However, DFMO co-treatment does not further increase PTS activity in the WM983B-BR cells, unlike that in the parental WM983B cells. If polyamine uptake (PTS activity) is increased in resistant BRAF$^{V600E}$ melanoma cells (made resistant by chronic treatment with PLX4720), then we predict that ***AP*** treatment will increase cell death in BRAFi-resistant BRAF$^{V600E}$ melanoma cells compared to those that are BRAFi-sensitive. However, although PTS activity was increased in BRAFi-resistant WM983B-BR cells, their sensitivity to ***AP*** was not significantly changed compared to that found in parental BRAFi-sensitive WM983B cells.

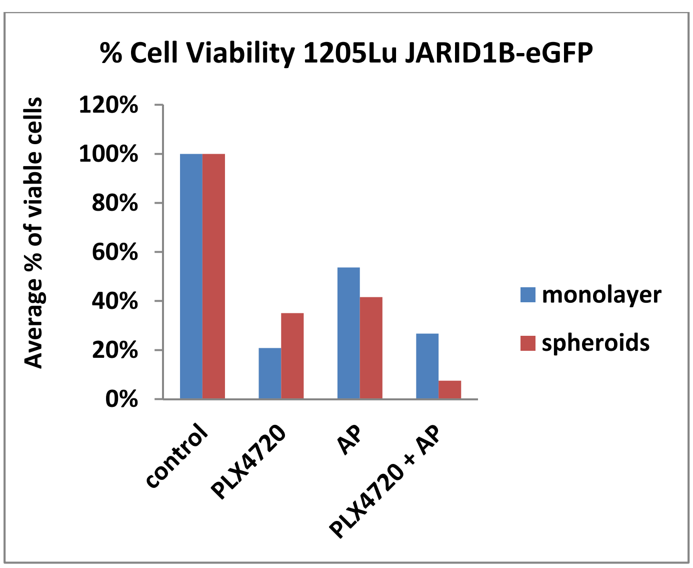
**Major Task 2:** To determine to what extent ***AP*** can eliminate CSC-like subpopulations that are enriched following chronic BRAF inhibition

Accumulating literature shows that treatment with BRAF inhibitors enriches a slow cycling cancer stem cell-like (CSC) subpopulation in melanomas. We proposed 2 different approaches (major tasks 2 and 3) to test our hypothesis that ***AP*** will accumulate selectively in BRAFi-resistant CSCs as a result of induced PTS activity. In task 2, we proposed to use BRAF$^{V600E}$ melanoma cells that have been chronically exposed to low dose PLX4720 (i.e. WM983B-BR cells) to examine the effect of ***AP*** on BRAFi-resistant CSCs. However, analyses of WM983B-BR cells by flow cytometry has not revealed increased expression of melanoma CSC markers such as JARID1B, CXCR4, CD271, or CD34 compared to that seen in parental WM983B cells. *It is* *likely that chronic treatment with PLX4720 leads to differentiation of the CSC subpopulation and loss of the enrichment for this stem cell-like subpopulation.* In this case, we predict that PLX4720 pulse treatment of BRAFi-sensitive melanoma cells (such as WM983B cells) will lead to the greatest enrichment of stem-cell-like subpopulations with greater formation of spheroids with increased expression of CSC markers and perhaps sensitivity to ***AP***. Indeed, as mentioned previously, the IC50 to ***AP*** was not significantly changed in WM983B-BR cells compared to WM983B cells. For these reasons, we have focused more on accomplishing goals in major task 3 where $BRAF^{V600E}$ melanoma cells are not chronically treated with low dose PLX4720 but are treated for a much shorter time with higher concentrations of PLX4720.

**Major Task 3:** To determine to what extent ***AP*** can overcome the enrichment of slow cycling, invasive $J/EGFP^{high}$ BRAF inhibitor-resistant melanoma cells and its correlation with PTS activity and CXCR4 signaling.

Since it has been shown that treatment with various chemotherapeutic drugs including PLX4720 enriches for a slow-cycling melanoma cell subpopulation that expresses the H3K4-demethylase JARID1B, we have used a cell-based reporter system to test whether this resistant phenotype can be eradicated with ***AP***. For isolation of live $JARID1B^{+}$ cells, we are now using two $BRAF^{V600E}$ mutant melanoma cell lines (WM3734 and 1205Lu) that have been stably transduced with a JARID1B-promoter-EGFP-reporter construct (cells obtained from Dr. Meenhard Herlyn, The Wistar Institute). For 3D cell culture we have used NanoCulture Plates (NCP, from SCIVAX Life Sciences) which is a scaffold-based 3D culture system in which cells can easily form spheroids. The spheroid microenvironment closely resembles that in tumors, and is more relevant for drug sensitivity compared to that seen with monolayer cultures. We have spent considerable amount of time optimizing the conditions to generate spheroids with $Wm3734^{JARID1Bprom\text{-}EGFP}$ cells and $1205Lu^{JARID1Bprom\text{-}EGFP}$ cells using the NCP culture method. We have found that melanoma cells growing as spheroids in NCP culture are more resistant to treatment with 25 µM PLX4720 compared to cells growing in monolayer. Confirming our hypothesis, we have found that short (2-3 day) exposure to PLX4720 leads to a 10 fold enrichment of JARID1B-driven GFP expressing melanoma cells, which is characteristic of a CSC subpopulation. We have been excited to find that 48 hr co-treatment with both 25 µM PLX4720 and 25 µM ***AP*** led to the largest reduction in cell viability as measured by a luminescent ATP assay in the spheroid cultures.

**Fig. 4. Increased Resistance of Spheroid Melanoma Cells to PLX4720 is Overcome with *AP* Co-Treatment**



**Major Task 4:** To evaluate to what extent ***AP*** will block macrophage-mediated resistance to treatment with BRAF-inhibitors

It was recently reported that factors produced by macrophages confer melanoma cell resistance to BRAF inhibition. We have obtained monocytes purified from human donor peripheral blood, and differentiated them to macrophages using human melanoma conditioned medium or by adding cytokines (GM-CSF or M-CSF). We have confirmed the observation that PLX4720 induces M2-macrophages (not M1 macrophages) to produce VEGF (Fig. 5). VEGF stimulates cell growth in melanoma cells by reactivating the MAPK pathway. We then co-cultured BRAF-inhibitor-sensitive 1205Lu$^{JARID1Bprom-EGFP}$ melanoma cells with differentiated human macrophages seeded on a collagen-coated Transwell filter (pore size: 0.4 μm). Using this human macrophage and melanoma cell co-culture system, we have shown that human M2 macrophages contribute to melanoma tumor cell resistance to the BRAFi, PLX4720 (Fig. 6). Whereas human monocytes differentiated to M2 macrophages using conditioned medium from 1205Lu melanoma cells increase the viability of PLX4720-treated 1205Lu melanoma cells, monocytes differentiated to M2 macrophages using M-CSF do not. We are repeating this co-culture assay with increased numbers of M2 macrophages to further increase the viability of the BRAFi-treated melanoma cells, and then to test to what extent ***AP*** will block this macrophage-mediated resistance of melanoma cells to PLX4720.

**Fig. 5. PLX4720 Induction of VEGF in Human M2 Macrophages**

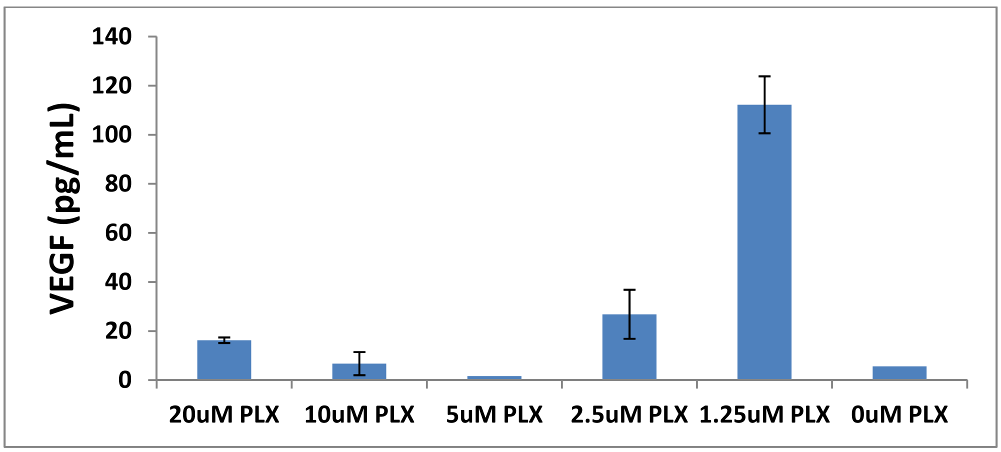
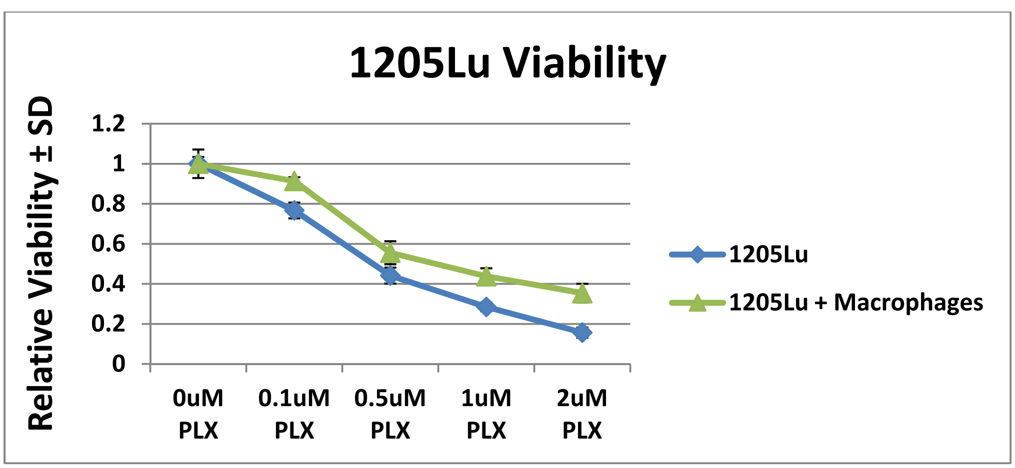



**Fig. 6. Macrophage-Mediated Resistance in PLX4720-Treated Melanoma Cells**

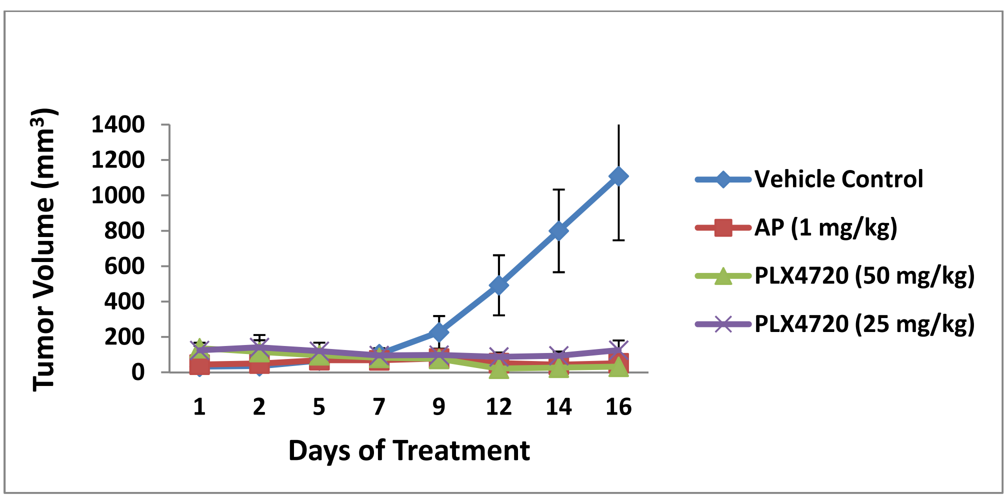


**Aim #2:** To evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth in mice following orthotopic injection of mutant BRAF melanoma cells

The *in vivo* dosing scheme of ***AP*** (also called Me44Nap44Me by my collaborator, Dr. Otto Phanstiel) was optimized for use in murine tumor studies. A similar evaluation was made using another competitive inhibitor of polyamine transport, Trimer44NMe, (see UCF consortium report at the end of this section). The Me44Nap44Me compound (***AP***) is a cytotoxic polyamine compound that enters and kills cells via the polyamine transport system. In contrast, the Trimer44NMe is much less toxic and effectively inhibits the uptake of native polyamines. The *in vivo* pharmacokinetics were unknown, and our studies were the first studies to begin delineating the pharmacokinetics of these compounds in mice and their tissue distribution and potential clearance pathways.

To measure blood levels of ***AP*** over time, non-tumor bearing C57Bl/6 mice (n = 3 mice/time point) were injected i.p. with 2 mg/kg ***AP*** in 100 µl sterile PBS, and then blood (0.2 to 0.3 ml) was collected from the facial vein following piercing with a goldenrod lancet at 0.5, 4, 8, 24, 48, and 72 hr after ***AP*** injection. When dosed alone without DFMO, we observed rapid clearance of ***AP*** (Me44Nap44Me) from the blood after 1 h, with likely clearance through the kidneys (see UCF consortium report). To develop a dosing scheme for multiple ***AP*** treatments in tumor experiments, C57Bl/6 mice (n = 3 mice/treatment group) were injected i.p. with 2 or 1 mg/kg ***AP*** every day in order to determine the maximum tolerated dose over a 2 week period of time. We found that mice demonstrated no adverse effects (no weight loss and no change in activity or outward appearance) with 2 weeks of daily i.p. injections of ***AP*** (1 mg/kg body weight). C57Bl/6 mice were then injected s.c. with syngeneic murine mutant BRAF, PTEN-null melanoma cells (YUMM1.7), and mice with established tumor xenografts were treated with ***AP*** (1 mg/kg, i.p., daily) or PLX4720 (25 or 50 mg/kg, oral gavage, bid). As expected, YUMM1.7 tumors rapidly regressed in mice treated with PLX4720. We were excited to see that daily ***AP*** treatment also suppressed tumor growth as effectively as the BRAFi, PLX4720 (Fig. 7). However, mice treated with ***AP*** began to lose weight after 2 weeks of treatment, and the experiment was terminated before we could evaluate if the tumors would develop resistance and begin growing again as was expected with PLX4720 treated mice. We also treated another group of mice s.c. injected with $BRAF^{WT}$ B16F10 melanoma cells with ***AP*** (1 mg/kg, i.p., daily) ± 0.5% (w/v) DFMO in the drinking water. Treatment with ***AP*** ± DFMO did not significantly inhibit tumor growth in the mice. However, we did observe that mice that were co-treated with ***AP*** and DFMO did not lose weight as did the mice that were treated with ***AP*** alone.

**Fig. 7. *AP* Treatment Retards Yumm1.7 Tumor Growth in Syngeneic Immunocompetent C57Bl/6 Mice**




Based on these experiments, we set up another group of mice s.c. injected with $BRAF^{V600E}$ YUMM1.7 melanoma cells and began treatment with PLX4720 in <u>all</u> mice having established tumors. One third of the mice were co-treated with ***AP*** (1 mg/kg, i.p., daily), and one third of the mice were co-treated with ***AP*** (1 mg/kg, i.p., daily) + 0.5% (w/v) DFMO in the drinking water. Figure 8 shows that tumors in mice treated with PLX4720 only rapidly shrunk in size initially, only to develop resistance to PLX4720 after 2 weeks of treatment to continue growing. We were able to co-treat mice with both ***AP*** and DFMO without any adverse effects (weight loss) in the mice and significantly slow down the recurrence of PLX4720-resistant tumors. Co-treatment with only ***AP*** was stopped when the mice began to lose weight, and this was accompanied with re-growth of the tumors despite continued PLX4720 treatment. With termination of ***AP*** treatment, the mice re-gained their lost weight. We are currently in the process of analyzing tumors, non-tumor-bearing skin, and various tissues from these mice for accumulation of ***AP*** by mass spectrometry and also for any pathology. Based on our preliminary pharmacokinetic findings with the Trimer44NMe PTI, we expect to see more accumulation of ***AP*** in tumor tissue when mice are co-treated with DFMO, which we believe may also afford more protection from adverse effects in normal tissues.

**Fig. 8. *AP* ± DFMO Treatment Delays the Recurrence of BRAF$^{V600E}$ Melanoma Tumors That Occurs in Animals Treated with PLX4720 Alone**

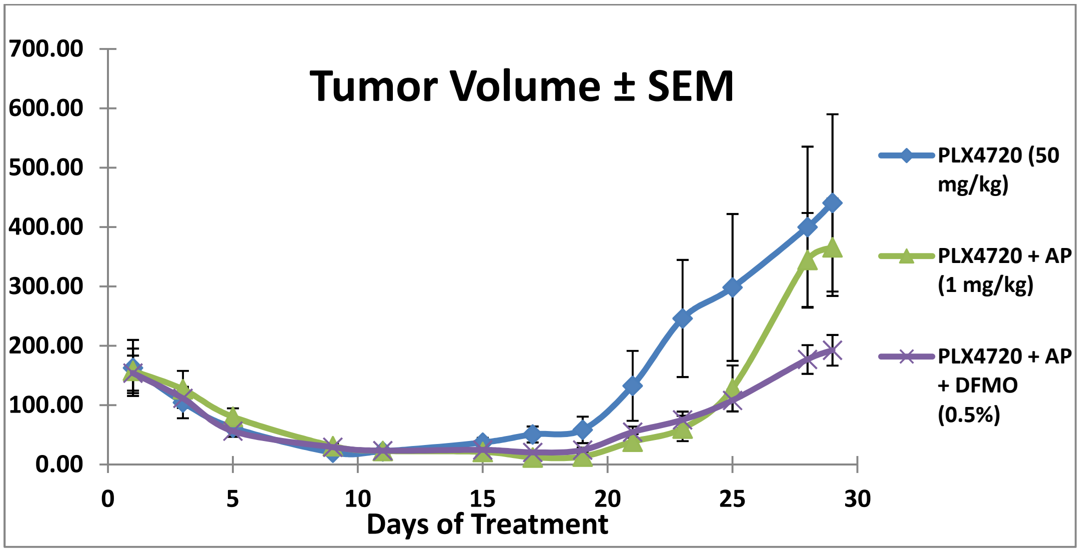
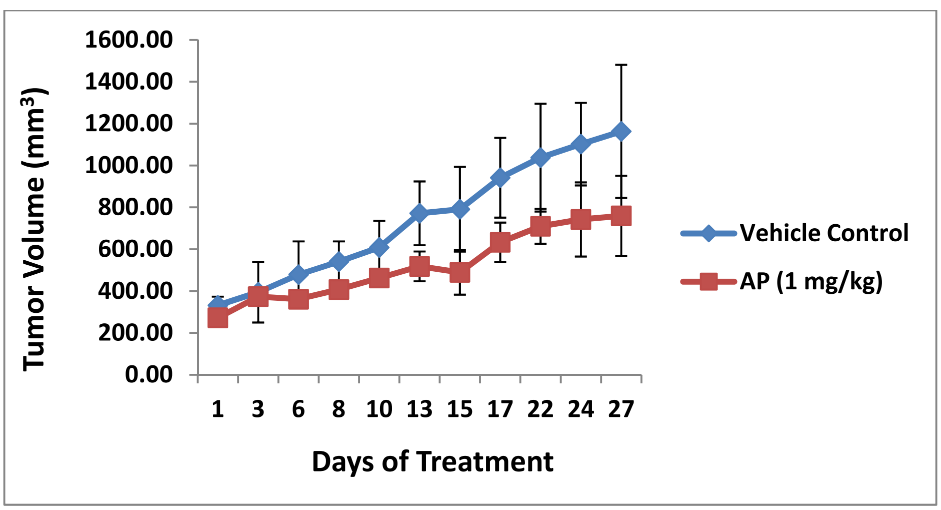



**Fig. 9. *AP* Treatment Retards Growth of Human WM938B Melanoma Xenografts in Athymic Nude (NCI) Mice**




We also did a pilot experiment to test the effect of ***AP*** treatment in athymic nude NCI mice s.c. injected with human WM983B melanoma cells (2 x $10^6$ cells in 50% Matrigel). We observed that ***AP*** treatment retarded tumor growth in these mice. Because of weight loss in ***AP*** treated mice, we had to reduce the ***AP*** treatment to every other day in the last week of treatment. Future tumor experiments in animals will include treatment with both ***AP*** and DFMO.

## Year 1 report – Activity in Phanstiel Laboratory at University of Central Florida

Several polyamine-targeting compounds developed at UCF were tested for their ability to target melanomas in mice at Lankenau Institute for Medical Research (LIMR). Two lead compounds, Trimer44NMe and Me44Nap44Me (Figure 1) were evaluated for their ability to target tumor tissues over normal skin and for their clearance rates from mice in order to inform future dosing regimens and routes of administration. Both compounds are competitive inhibitors of polyamine transport. ***AP***, the Me44Nap44Me compound, is a cytotoxic polyamine compound that enters and kills cells via the polyamine transport system.[1] In contrast, the Trimer44NMe is much less toxic and effectively inhibits the uptake of native polyamines.[2] The *in vivo* pharmacokinetics were unknown and these are the first studies to begin delineating the pharmacokinetics of these compounds in mice and their tissue distribution and potential clearance pathways.

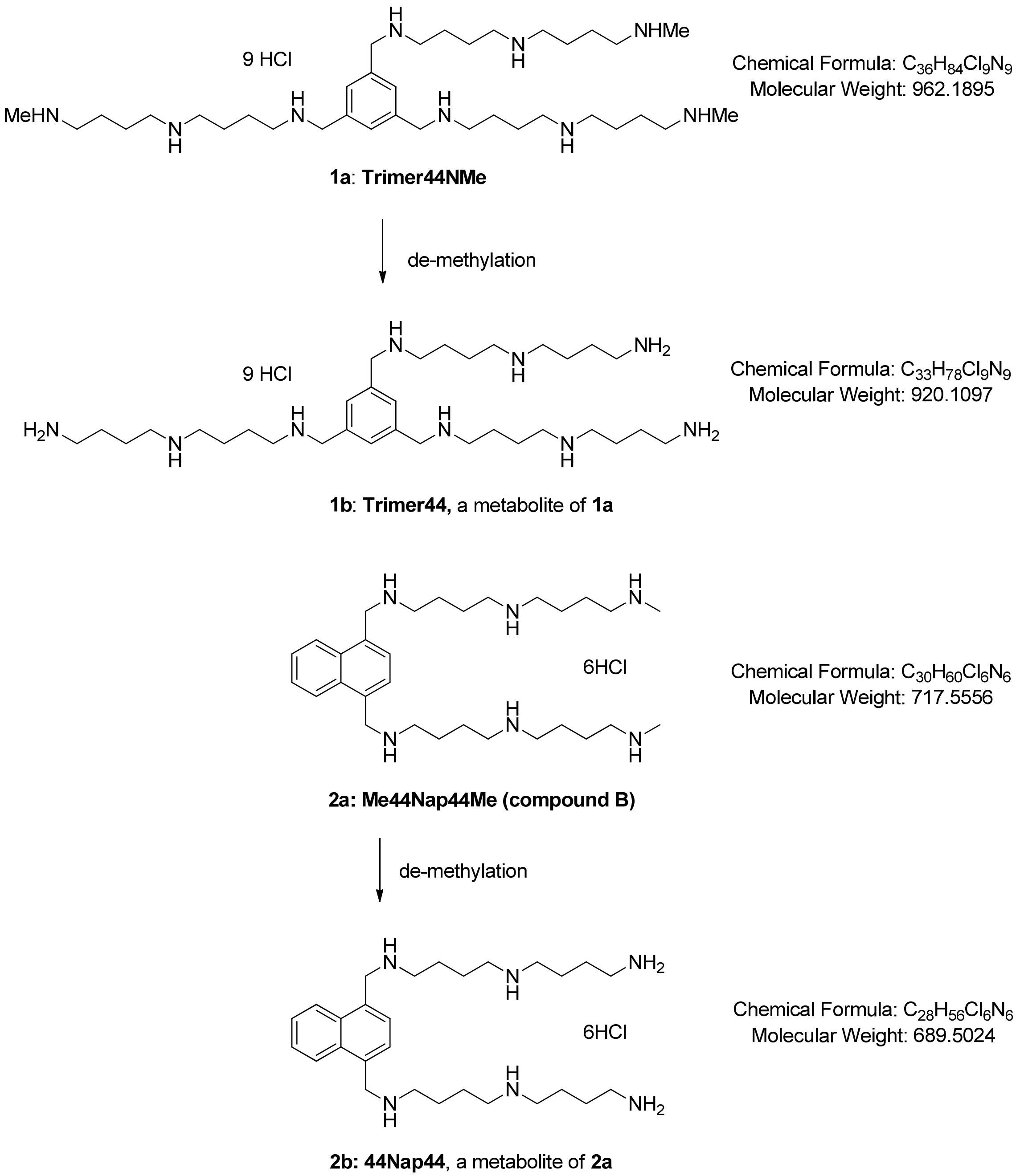



**Figure 1**. Structures of Trimer44NMe (**1a**) and Me44Nap44Me (**2a**) and their de-methylated metabolites **1b** and **2b**, respectively.

**Results and Discussion.** We received numerous samples (both plasma samples and frozen, finely ground tissue samples from treated mice) from Dr. Susan Gilmour at the Lankenau Institute for Medical Research (LIMR) this past year. These were processed here at UCF and the levels of protein and the native polyamines were determined and the data was reported in nmol polyamine/ mg protein in the following Tables.

We began by comparing two methods for polyamine level determination. The first was our established N-dansylation protocol using HPLC retention times to identify each polyamine. Using established calibration curves with pure standards, we were able to quantify each of the native polyamines (Table 1). In addition, we used a LC-MS technique to also measure native polyamine levels through a service provided by the metabolomics core at Sanford Burnham (SB). In this method, the protein levels were determined at UCF by the BCA protein determination method (Pierce) and the polyamine levels determined at SB by LC-MS. The results were then normalized and the polyamine levels were reported as nmol polyamine/mg protein. The results are shown in Table 2. The mass spectrometry method looks directly at the native polyamines, whereas the HPLC method derivatizes the native polyamines into N-dansylated compounds, which are highly fluorescent and can be detected by the fluorescence detector on the HPLC. As shown in Table 1 (HPLC data) and Table 2 (LC-MS data), similar results were obtained via the two methods. This provided a nice cross check of our methods and showed similar results, thus, validating our approach.

**Table 1**. HPLC Dansylation method results[a]

| **Description** | **LIMR code** | **Putrescine nmol/mg protein** | **Spermidine nmol/mg protein** | **Spermine nmol/mg protein** | **Total Polyamines nmol/mg protein** |
|---|---|---|---|---|---|
| Skin Samples Control | Animal 9143-9145, Control-1 Skin | 0.25 ± 0.05 | 3.61 ± 1.24 | 1.56 ± 0.45 | 5.41 ± 1.65 |
| Skin Samples PBT | Animal 9146-9149, PBT-1 Skin | 0.15 ± 0.21 | 2.06 ± 0.56 | 1.35 ± 0.36 | 3.55 ± 1.06 |
| Tumor Samples Control | Animal 9143-9145, Control-1 Tumor | 6.19 ± 0.70 | 24.00 ± 7.15 | 9.03 ± 2.13 | 39.22 ± 9.85 |
| Tumor Samples PBT | Animal 9146-9149, PBT-1 Tumor | 2.26 ± 0.53 | 20.98 ± 6.28 | 11.27 ± 3.10 | 34.51 ± 9.34 |

[a]errors expressed as the standard deviation around the reported mean.

**Table 2.** Mass Spectrometry LC-MS method results[a]

| Description | LIMR code | Putrescine nmol/mg protein | Spermidine nmol/mg protein | Spermine nmol/mg protein | Total Polyamines nmol/mg protein |
|---|---|---|---|---|---|
| Skin Samples Control | Animal 9143-9145, Control-1 Skin | 0.78 ± 0.38 | 3.87 ± 2.14 | 1.75 ± 0.86 | 6.40 ± 3.36 |
| Skin Samples PBT | Animal 9146-9149, PBT-1 Skin | 0.22 ± 0.10 | 2.67 ± 0.84 | 1.86 ± 0.56 | 4.74 ± 1.47 |
| Tumor Samples Control | Animal 9143-9145, Control-1 Tumor | 4.60 ± 0.12 | 11.73 ± 1.96 | 8.30 ± 1.91 | 24.63 ± 3.74 |
| Tumor Samples PBT | Animal 9146-9149, PBT-1 Tumor | 2.46 ± 0.74 | 13.84 ± 4.37 | 13.17 ± 4.42 | 29.47 ± 8.47 |

[a]errors expressed as the standard deviation around the reported mean.

**Table 3**. Mass spectrometry LC-MS method results to quantify the Trimer44NMe PTI [a]

| Description | LIMR code | Trimer44NMe nmol/mg protein |
|---|---|---|
| Skin Samples Control | Animal 9143-9145, Control-1 Skin | 0.00 ± 0.00 |
| Skin Samples PBT | Animal 9146-9149, PBT-1 Skin | 0.03 ± 0.03 |
| Tumor Samples Control | Animal 9143-9145, Control-1 Tumor | ND |
| Tumor Samples PBT | Animal 9146-9149, PBT-1 Tumor | 1.02 ± 0.39 |

[a]errors expressed as the standard deviation around the reported mean.

Armed with these methods we measured samples (plasma or frozen, finely ground tissue samples) as they arrived from mouse experiments conducted at the Lankenau Institute for Medical Research (LIMR, Wynnewood, PA). Plasma and tissue samples were from mice treated with polyamine blockade therapy (PBT, consisting of 0.5% difluoromethylornithine [DFMO] in the drinking water plus daily i.p. injections of Trimer44NMe [3 mg/kg body weight] polyamine transport inhibitor [PTI]) or from mice treated with control vehicle. For example, we investigated the distribution of the Trimer44NMe polyamine transport inhibitor (PTI) compound. As shown in **Table 3**, a marked preference was observed for targeting the tumor over the peripheral skin. This is important as it suggests that the Trimer44NMe PTI compound may deposit in the tumor tissues, at least when mice are co-treated with both the Trimer44NMe and DFMO (PBT).

**Table 4.** Plasma Levels of Trimer44NMe PTI and its de-methylated metabolite, Trimer44 (expressed in nmol/mg protein)

| DFMO (1 mM) | Dose, Route of Administration | Time After Trimer dose Blood Collected | Trimer44 (nmol/mg protein) | Trimer44NMe (nmol/mg protein) |
|---|---|---|---|---|
| Yes | 3 mg/kg, oral gavage | 30 min | 0.21 | 0.92 |
| Yes | 3 mg/kg, oral gavage | 2 hr | 0.04 | 0.14 |
| Yes | 3 mg/kg, IP inj | 30 min | ND | 0.35 |
| Yes | 3 mg/kg, IP inj | 2 hr | 0.03 | 0.13 |
| No | 6 mg/kg, oral gavage | 30 min | 0.04 | 0.26 |
| No | 6 mg/kg, oral gavage | 2 hr | ND | ND |

[a]errors expressed as the standard deviation around the reported mean. Note: Trimer44 is a metabolite of Trimer44NMe which occurs via demethylation of Trimer44NMe.

As shown in Table 4, low levels of the Trimer44 metabolite were observed when the Trimer44NMe PTI compound was given via i.p. injection in the presence of DFMO. Higher levels of the trimer44 metabolite and trimer44NMe parent compound were observed when dosed via oral gavage at 3mg/kg in the presence of DFMO. Interestingly, lower levels of Trimer44NMe and its metabolite were noted when dosed at 6 mg Trimer44NMe/kg-mouse via oral gavage in the absence of DFMO. We concluded from this study that the

Trimer44NMe compound is orally bioavailable and that its uptake via oral administration is increased significantly in the presence of DFMO, which is also dosed orally.

We also looked at the plasma levels of Trimer44 and Trimer44NMe, when Trimer44NMe is given i.p. at 3 mg/kg. (i.e., Bag A studies). The results are shown in Table 5.

**Table 5.** Time course study of the plasma levels of Trimer44NMe PTI and its metabolite Trimer44 after i.p. injection (3 mg/kg).[a]

| Time (hour) | Putrescine (nmol/mg protein) | Spermidine (nmol/mg protein) | Spermine (nmol/mg protein) | Trimer44 (nmol/mg protein) | Trimer44NMe (nmol/mg protein) |
|---|---|---|---|---|---|
| 0.5 | 0.63 ± 0.93 | 6.64 ± 6.02 | 0.50 ± 0.36 | 0.06 ± 0.03 | 1.48 ± 0.27 |
| 1 | 4.29 ± 6.06 | 4.20 ± 4.50 | 0.57 ± 0.35 | ND | 0.58 ± 0.83 |
| 2 | 0.45 ± 0.76 | 2.75 ± 2.64 | 0.30 ± 0.23 | ND | 0.11 ± 0.10 |
| 4 | 0.20 ± 0.24 | 2.89 ± 0.85 | 0.46 ± 0.23 | ND | 0.45 ± 0.06 |
| 6 | | | | ND | ND |
| 24 | | | | ND | ND |

[a]ND = not detected; whereas a blank entry means experiment was not done.

While there was considerable variability in these experiments, the data suggested that approximately 66% of the Trimer44NMe is cleared after 3.5 h (compare 0.5 h vs 4 h timepoints) giving the Trimer44NMe PTI an approximate half life of approximately 3 hours in circulating blood plasma.

We also looked at tissue distribution of ***AP*** (Me44Nap44Me) when tumor-bearing mice were injected i.p. at 1 mg/kg every day for 2 weeks. The results are shown in Table 6.

**Table 6**. Tissue Distribution of Native Polyamines and ***AP*** (Me44Nap44Me) when Me44Nap44Me is dosed i.p. at 1 mg/kg[a]

| Tissue type | Putrescine nmol/mg protein | Spermidine nmol/mg protein | Spermine nmol/mg protein | 44Nap44 nmol/mg protein | MeN44Nap44Nme (nmol/mg protein) |
|---|---|---|---|---|---|
| Tumor | 9.95 ± 5.66 | 6.49 ± 1.79 | 0.22 ± 0.05 | ND | ND |
| Skin | 2.46 ± 1.50 | 7.29 ± 3.78 | 0.45 ± 0.32 | 0.01[b] | 0.04[b] |
| Spleen | 3.42 ± 1.41 | 3.12 ± 0.60 | 0.15 ± 0.06 | ND | ND |
| Kidney | 4.20 ± 0.85 | 11.11 ± 1.84 | 0.91 ± 0.16 | 0.01 | 0.04 ± 0.01 |

[a] errors expressed as the standard deviation around the reported mean. [b] only observed in one sample, the rest were not detected (ND)

Table 6 suggested that ***AP*** (Me44Nap44Me) is rapidly cleared as no appreciable amounts were found in various tissues. Since all four of the kidney samples contained trace quantities of ***AP*** (Me44Nap44Me), we suspect that this water-soluble compound is likely cleared through the kidney.

**Table 7.** Time course blood plasma levels of the native polyamines and ***AP*** (Me44Nap44Me) and its metabolite 44Nap44 after C57Bl6 mice were injected i.p. with 2 mg/kg ***AP*** (Me44Nap44Me) without any DFMO co-treatment.

| Time (h) | Putrescine nmol/mg protein | Spermidine nmol/mg protein | Spermine nmol/mg protein | 44Nap44 nmol/mg protein | MeN44Nap44Nme (nmol/mg protein) |
|---|---|---|---|---|---|
| 0.5 | 0.30 ± 0.13 | 4.07 ± 1.21 | 0.18 ± 0.01 | 0.02 ± 0.00 | 0.42 ± 0.14 |
| 1 | 1.40 ± 2.04 | 3.94± 4.45 | 0.16 ± 0.19 | 0.00 | 0.33 ± 0.19 |
| 2 | 0.00 ± 0.01 | 0.08 ± 0.03 | 0.00± 0.00 | 0.00 | 0.00 |
| 4 | 0.00 ± 0.00 | 0.43 ± 0.08 | 0.04 ± 0.01 | 0.00 | 0.00 |
| 24 | | | | 0.00 | 0.00 |

[a] errors expressed as the standard deviation around the reported mean.

Table 7 suggests that only small amounts of ***AP*** (Me44Nap44Me) are still present in the plasma at the 0.5 h and one hour time points and are not detected at the 2h and 4h time points; suggesting that ***AP*** (Me44Nap44Me) is rapidly cleared from the blood.

**Conclusions.** In summary, the assays were validated by another technique and used to assess the two polyamine compounds *in vivo*. The highlights thus far are that the Trimer44NMe compound seems to target the tumor selectively when animals are co-treated with both Trimer44NMe and DFMO, and that the Trimer44NMe is orally bioavailable especially in the presence of DFMO. When administered together with DFMO, the Trimer44NMe PTI has an approximate half-life of 3 hours in circulating blood plasma. When dosed alone without DFMO, we observed rapid clearance of ***AP*** (Me44Nap44Me) from the blood after 1 h with likely clearance through the kidneys. Because DFMO treatment upregulates the polyamine transport system in tumors which then increases uptake of either the Trimer44NMe or ***AP*** (Me44Nap44Me) into tumors, we plan to evaluate the tissue distribution and clearance of ***AP*** (Me44Nap44Me) in tumor-bearing mice following co-treatment with DFMO and ***AP***.

## References


1. Muth, A.; Kamel, J.; Kaur, N.; Shicora, A. C.; Ayene, I. S.; Gilmour, S. K.; Phanstiel, O., Development of polyamine transport ligands with improved metabolic stability and selectivity against specific human cancers. *J Med Chem* **2013,** *56* (14), 5819-5828.

2. Muth, A.; Madan, M.; Archer, J. J.; Ocampo, N.; Rodriguez, L.; Phanstiel, O., Polyamine transport inhibitors: design, synthesis, and combination therapies with difluoromethylornithine. *J Med Chem* **2014,** *57* (2), 348-363.

**What opportunities for training and professional development has the project provided?**

Nothing to report.

**How were the results disseminated to communities of interest?**

I gave a lay presentation concerning my research to members of the Lankenau Women's Board, a dedicated group of women who have raised impressive amounts of monies to support the Lankenau Medical Center.

**What do you plan to do during the next reporting period to accomplish the goals?**

**Aim 1:** To compare the effect of increasing concentrations of ***AP*** with long-term and pulse BRAFi (PLX4720) co-treatment a) on the enrichment of BRAFi-resistant slow cycling CSC melanoma subpopulations and b) in melanoma tumor cell survival in macrophage co-culture assays ± PLX4720.

Using some of the melanoma cell lines that we have characterized for their PTS activity and sensitivity to ***AP*** and PLX4720, we plan to continue comparing the effect of ***AP*** on BRAF mutant melanoma cells that are BRAFi-sensitive or BRAFi-resistant with spheroid formation in ultra-low attachment plates, 3-dimensional spheroid growth/invasion/survival using NCP plates, and invasiveness through collagen-coated Transwell filters towards the chemoattractant SDS-1. However, we predict that PLX4720 <u>pulse</u> treatment of BRAFi-sensitive melanoma cells will lead to the greatest enrichment of stem-cell-like subpopulations with greater formation of spheroids with increased invasiveness and increased expression of CXCR4. We predict that ***AP*** co-treatment with this PLX4720 pulse treatment will inhibit this enrichment of these putative MICs (slow cycling JARID1B$^{High}$ subpopulation). We will compare FACS-sorted J/EGFP$^{high}$ and J/EGFP$^{low}$ subpopulations for PTS activity ([$^{3}$H] spermidine uptake), for CXCR4 expression and signaling targets (by immunoblot assay), and for invasiveness across matrigel-coated filters. In addition, we will use our human macrophage/melanoma co-culture model to test to what extent ***AP*** can block macrophage-induced PLX4720 resistance in the melanoma cells assaying for % viability of the melanoma tumor cells and for levels of phosphorylated ERK, STAT3, VEGF secretion, and CXCR4.

**Aim 2.** To evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth and metastasis in mice following orthotopic injection of mutant BRAF melanoma cells.

Building on our findings with ***AP*** and PXL4720 dosing and initial tumor experiments in mice, we plan to initiate additional animal tumor studies using human mutant BRAF melanoma cells WM983B and TGL-labeled BRAF inhibitor-resistant WM983B-BR cells in athymic nude mice. Based on our previous studies and future expected results of tissue distribution of ***AP*** in mice treated with ***AP*** ± DFMO, we plan to treat mice with ***AP*** (1 mg/kg, i.p., daily) + 0.5% (w/v) DFMO in the drinking water with or without PLX4720 (supplemented in the chow (417 mg/kg). In addition, we plan to determine to what extent the combination of ***AP*** and PXL4720 may overcome BRAFi-resistance and promote tumor killing using the YUMM1.7 melanoma model in immunocompetent C57Bl/6 mice. It has been reported that the initial period of remission produced by BRAFi (PLX4720) treatment provides an opportunity to activate an immune attack that can prevent the recurrence of BRAFi-resistant melanoma. Because we have recently shown that combination treatment of DFMO + Trimer44NMe PTI enhances anti-tumor immune response, we predict that a polyamine blocking therapy with ***AP*** + DFMO may also activate an immune response that can suppress recurrence of aggressive malignant melanoma following treatment with a BRAF inhibitor. We predict that co-treatment with ***AP*** + DFMO will not only increase the anti-tumor effects of PLX4720 but also diminish infiltrating tumor associated myeloid cells. If ***AP*** is acting primarily by blocking CXCR4 in both metastatic tumor cells and infiltrating macrophages, then we will compare with the anti-metastatic activity of co-treatment with PLX4720 and the CXCR4 inhibitor, AMD3100 (1.25 mg/kg s.c. every other day). Finally, our goal is to prepare figures and manuscript(s) to report results of this study. Our studies will provide an improved understanding of how mutant BRAF-driven melanomas escape BRAF inhibitors as well as a novel therapy which can seek out and destroy tumor cells which escape these current chemotherapeutic drugs.

## 4. IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

The polyamine transport system (PTS) is not well characterized even though we know that it is often upregulated in tumors. We have discovered that PTS activity is increased in melanoma tumor cells possessing a BRAF mutation. We have created a novel drug ***AP*** that is disguised as a polyamine to enter tumor cells that have increased PTS activity, and then kills the tumor cells. In contrast, normal cells have limited PTS activity and will remain unharmed at the low doses of ***AP*** needed to kill the tumor cells. Our work has greatly added to our understanding of the PTS in tumors and how we can harness this transport system to better treat resistant forms of melanoma.

**What was the impact on other disciplines?**

We have been optimizing techniques to identify how a polyamine-targeting drug (***AP***) can selectively target and kill cancer stem cell populations in melanomas and also possibly reverse the ability of macrophages in the melanoma tumor microenvironment to make melanoma tumor cells resistant to certain chemotherapy drugs. So our work impacts other disciplines such as cancer stem cells and the immune response in tumors.

**What was the impact on technology transfer?**

We have invented novel polyamine-derived drugs (including ***AP***) that inhibit the uptake of polyamines by virtue of their polyamine tails that compete with normal polyamines for entry into tumor cells via the polyamine transport system (PTS). We have filed a provisional patent that claims that these novel polyamine-derived drugs (including ***AP***) can modulate the immune response to tumors.

**What was the impact on society beyond science and technology?**

The impact of this project will be a new therapy for melanoma patients that will prevent tumor relapse and improve survival. New medicines provide physicians with new options for patient care.

## 5. CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

Nothing to report.

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

**Changes that had a significant impact on expenditures**

Nothing to report.

**Significant changes in used or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

**Significant changes in use or care of human subjects**

Nothing to report.

**Significant changes in use or care of vertebrate animals**

Nothing to report.

**Significant changes in use of biohazards and/or select agents**
Nothing to report.

## 6. PRODUCTS:

**Publications, conference papers, and presentations**

**Journal publications**
We acknowledged partial support from this DOD grant for a manuscript that was recently accepted by Oncotarget: "A novel polyamine blockade therapy activates an anti-tumor immune response" by Eric Alexander, Allyson Minton, Molly Peters, Otto Phanstiel, and Susan Gilmour.

**Books or other non-periodical, one-time publications**
Nothing to report.

**Other publications, conference papers, and presentations**
Our work was presented in an invited oral presentation and also in a poster presentation at the Polyamines Gordon Research Conference in June, 2017.

**Website(s) or other Internet site(s)**
Nothing to report.

**Technologies or techniques**
Nothing to report.

**Inventions, patent applications, and/or licenses**
We have invented and characterized novel polyamine-derived drugs (including ***AP***) that inhibit the uptake of polyamines by virtue of their polyamine tails that compete with normal polyamines for entry into tumor cells via the polyamine transport system (PTS). We have filed a provisional patent (joint patent by UCF and LIMR) that claims that these novel polyamine-derived drugs (including ***AP***) can modulate the immune response to tumors.

**Other products**
We are developing a novel new therapy for melanoma patients that will prevent tumor relapse and improve survival.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | Susan Gilmour |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | 0000-0001-6228-860X |
| Nearest person month worked: | 3 |
| Contribution to Project: | Dr. Gilmour has developed experimental designs, analyzed and interpreted data, written all IACUC protocols, directly supervised Allyson Minton, Hayley Schupp, and Amy Bendell in her laboratory, coordinated sharing of resources with the lab of Meenhard Herlyn at the Wistar Institute, and coordinated sending blood and tissue samples to the lab of Dr. Otto Phanstiel at the |

University of Central Florida for analyses of ***AP*** and polyamine levels, written progress reports and prepared poster presentation , and been responsible for working out technical problems for many of the proposed experimental procedures and for all histopathologic analysis.

Funding Support: There is no other funding support for this project other than the DoD.

Name: Allyson Minton
Project Role: Biomedical Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6
Contribution to Project: Ally Minton expanded human and murine melanoma cells, made frozen stocks, determined $IC_{50}$s for PLX4720 and ***AP***, and assayed cells for PTS activity. She also determined the dosing schedule for *in vivo* experiments with ***AP*** and performed tumor experiments in mice with ***AP*** ± PLX4720. She left in February 2017 and was replaced by Haley Schupp.
Funding Support: There is no other funding support for this project other than the DoD.

Name: Haley Schupp
Project Role: Biomedical Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Haley Schupp determined $IC_{50}$s for PLX4720 and ***AP*** and assayed melanoma cells for PTS activity. She left in May 2017 and was replaced by Amy Bendell.
Funding Support: There is no other funding support for this project other than the DoD.

Name: Amy Bendell
Project Role: Post Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Dr. Bendell compared the effect of increasing concentrations of ***AP*** with long-term and pulse BRAFi (PLX4720) co-treatment a) on the enrichment of BRAFi-resistant slow cycling CSC melanoma subpopulations and b) in melanoma tumor cell survival in macrophage co-culture assays ± PLX4720.
Funding Support: There is no other funding support for this project other than the DoD.

Name: Otto Phanstiel
Project Role: Subrecipient PI
Researcher Identifier: 0000-0001-7101-1311
Nearest person month worked: 0.1 cal month
Contribution to Project: Provided training and supervision of the technician
Funding Support: There is no other funding support for this project other than the DoD.

Name: Chelsea Massaro
Project Role: Technician
Nearest person month worked: 12 cal months
Contribution to Project: Processed biological samples received from Lankenau Institute and determined both polyamine levels and levels of the polyamine transport targeting compound
Funding Support: There is no other funding support for this project other than the DoD.

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Yes.

**Susan Gilmour changes in other support**

**Active grant that ended**

**Title:** A Novel and Selective Treatment for Triple-Negative Breast Cancer
**PI:** Gilmour, S.K.
**Supporting Agency:** The Women's Board of Lankenau Medical Center
**Performance Period:** 7/1/15-6/30/16
**Level of effort:** As needed (No salary is requested for Dr. Gilmour)
**Project's Goals:** The major goal of this project is to characterize a new drug which selectively enters and kills triple negative (TN) breast cancer via their up-regulated polyamine transport system (PTS).

**Pending grant that was funded**

None.

**New active grants**
**Title:** Studies on the Use of Dabigatran to Block Cancer Progression
**PI:** Gilmour, S.K.
**Supporting Agency:** Boehringer Ingelheim International GmbH
**Performance Period:**11/1/16-10/31/17
**Level of effort:** 10%
**Level of funding:** $70,200 direct costs
**Project's Goal**: The major goal of this project is to augment the efficacy of immune checkpoint inhibitors with a combination of dabigatran etexilate and cisplatin.

**Title:** Development of a Novel Combination Therapy for Ovarian Cancer
**PI:** Gilmour, S.K
**Supporting Agency:** Sharpe-Strumia Research Foundation
**Performance Period:** 7/1/17-6/30/18
**Level of effort:** 5%
**Level of funding:** $45,000 direct costs
**Project's Goal**: The major goal of this study is to examine that increased levels of polyamines in ovarian tumors bolster PARP-mediated DNA repair, and that polyamine-depletion therapy will enhance ovarian tumor sensitivity to PARP inhibitors and platinum-based chemotherapy.

**Otto Phanstiel changes in other support**

CURRENT AWARD

1. Title: Targeting Increased Polyamine Transport of Resistant Melanomas
   PI: Gilmour, Susan; Co-Investigator: Phanstiel, Otto
   Time Commitment: 1% effort
   Supporting Agency: DOD
   Contracting/Grants Officer: Wendy Baker DOD
   Performance Period: 08/01/2016 – 07/31/2018
   Level of Funding: $37,042 Annual Direct Costs
   Project Goals: The goal of this project is to evaluate the performance of arypolyamines (***AP***) in treating melanomas
   Overlap: Not Applicable. This is the current award.

NEWLY AWARDED

2. Title: Targeting polyamine metabolism in the malaria parasite
   PI: Phanstiel, Otto; with co-PI Debopam Chakrabarti
   Time Commitment: 1% effort
   Supporting Agency: UCF College of Medicine
   Contracting//Grants Officer: Not Applicable/Internal Funds
   Performance Period: 01/01/2017 – 12/31/2017
   Level of Funding: $15,000 Total
   Project Goals: The goal is to investigate the role of polyamine metabolism on the proliferation of the malarial parasite
   Overlap: This project uses a combination of a polyamine transport inhibitor and a polyamine biosynthesis inhibitor to control the growth of the parasite *in vivo*. No overlap.

3. Title: Cognitive Functioning in Celiac Disease
   PI: Phanstiel is a collaborating investigator
   Time Commitment: 1%
   Supporting Agency: UCF College of Medicine
   Contracting/Grants Officer: Not Applicable/Internal Funds
   Performance Period: 01/01/2017 – 12/31/2017
   Level of Funding: $12,500
   Project Goals: The goal is to develop an improved understanding of how gut microflora and polyamines play a role in celiac disease.
   Overlap: no overlap

NEWLY PENDING

1. Title: Altered Polyamine Metabolism and Exchange Lead to Immune Privilege in Pancreatic Ductal Adenocarcinoma
   PI: Phanstiel, Otto
   Supporting Agency: National Institutes of Health
   Contracting/Grants Officer: Not Assigned Yet
   Performance Period: 04/01/2018 – 03/31/2023
   Level of Funding: $2,353,519
   Level of Effort: 25%
   Project Goals: The project will contribute to fundamental knowledge of how pancreatic cancers maintain immune privilege via altered polyamine metabolism and polyamine exchange with other cell types in the tumor microenvironment.

Overlap: none as this pending grant will look at pancreatic cancers and will focus on a polyamine depletion strategy.

2. Title: Smart antibiotic adjuvants for treating pathogenic Gram-negative bacteria
   PI: Phanstiel, Otto
   Supporting Agency: National Institutes of Health
   Contracting/ Grants Officer: Not Assigned Yet
   Performance Period: 04/01/2018 – 03/31/2021
   Level of Funding: $1,170,466
   Level of Effort: 25%
   Project Goals: This project will develop new compounds to serve as adjuvants for existing antibiotics.
   Overlap: none

NEWLY COMPLETED

1. Title: Development of Polyamine Transport Inhibitors for Treating Pancreatic Cancers
   PI: Phanstiel, Otto
   Supporting Agency: University of Central Florida (UCF) College of Medicine
   Contracting/Grants Officer: N/A Internal Award
   Performance Period: 1/01/2016 – 08/01/2016
   Level of Funding: $5,000
   Level of Effort: 1% Effort
   Project Goals: The goal of this project is to develop new non-polyamine base polyamine transport inhibitors for use in combination therapies with a polyamine biosynthesis inhibitor.
   Overlap: None. This UCF funded project is developing a new drug design predicated upon non-polyamine based platforms.

2. Title: I-Corps: Orlando Pharmaceuticals
   PI: Phanstiel, Otto
   Supporting Agency: National Science Foundation; Administered by UCF
   Contracting/Grants Officer: Deidre Coates, 4201 Wilson Blvd, Arlington, VA 22230, 703-292-4804
   Performance Period: 01/15/2016 – 07/15/2016
   Level of Funding: $2,550
   Level of Effort: 1%
   Project Goals: This NSF project trains students and professor team how to start new businesses and provides monies to create a spin-off start-up company, Orlando Pharmaceuticals, and covers customer interviews, prototype construction and travel costs.
   Overlap: None

**What other organizations were involved as partners?**

*Organization Name:* University of Central Florida
*Location of Organization:* Orlando, FL
*Partner's contribution to the project:* Synthesis of ***AP*** and quantification of levels of native polyamines and ***AP*** in biological samples

**9. APPENDICES:** None.